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Glycosylphosphatidylinositol from Apicomplexan protozoa

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2009

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A supplementary dissertation submitted to obtain a
Doctor of Philosophy degree in Biomedical Sciences,
Parasitology speciality

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Réponse immunitaire et développement chez les insectes

2009

Susana Filipa Garcia Ramos was awarded with a PhD grant (SFRH/BD/12210/2003) by the Fundação para a Ciência e Tecnologia, Ministério da Ciência Tecnologia e Ensino Superior

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Acknowledgments

I would like to sincerely thank to Prof. Dr. Henrique Silveira, for all the support and understanding, for supervising and revising this dissertation.

I would also like to thank to Dr. Fátima Nogueira, for correcting this dissertation and for all the suggestions, friendship and availability.

Glycosylphosphatidylinositol anchors

Many eukaryotic proteins are present at the cell surface embedded in the plasma membrane. For most of them, insertion in the membrane is accomplished by an anchor composed of carbohydrates and lipids, known as Glycosylphosphatidylinositol (GPI) anchors.

These molecules anchor several proteins to the membrane of eukaryotic cells. The anchor consists of a glycolipid structure that is added as a post-translational modification to the C terminus of many proteins. The lipid content of the molecule facilitates the anchoring at the phospholipid bilayer.

The GPI anchor is broadly distributed among eukaryotic organisms, including protozoa, fungi, plants, insects and mammals (Nosjean *et al.*, 1997). GPIs are not present in Eubacteria, but interestingly, GPI-anchored proteins have been detected in some Archaeobacteria, such as a strain from the genus *Sulfolobus*, which has been considered to be closely related to eukaryotes (Kobayashi *et al.*, 1997). GPI-anchored proteins are involved in several processes, and have important roles in signal transduction, lipid raft partitioning, prion disease pathogenesis, immune responses, cellular communication, apical membrane targeting and the pathobiology of trypanosomal parasites (Paulick & Bertozzi, 2008).

Glycosylphosphatidylinositol anchors were first characterized approximately 20 years ago. In 1976, Ikezawa *et al.* purified a phospholipase (type C – PI-PLC) enzyme from *Bacillus cereus* that was found to release alkaline phosphatase from rat kidney slices. In the following years several PLCs with similar function were purified from other bacteria, and, accordingly, other proteins were found to be released upon treatment with these enzymes. As phospholipase enzymes were known to act upon phosphatidylinositol molecules, the released proteins were suggested to be covalently attached to the cell membrane via a site on the protein and a phosphatidylinositol molecule embedded in the lipid bilayer (reviewed by Low, 1989; Figure I.1).

In 1985, Ferguson *et al.* found that the C terminus of the variant surface glycoprotein (VSG) of the parasite *Trypanosoma brucei* contained: i) an ethanolamine amide-linked to the C-terminal aminoacid, ii) a polysaccharide consisting of mannose, glucosamine and variable amounts of Galactose, and iii) a phospholipid that could be degraded by bacterial PLC generating 1,2-dimyristyl

glycerol. At the same time, Tse *et al.* (1985) found that the C-terminal cysteine of Thy-1, a glycoprotein expressed on mammalian thymocytes and the brain, was attached to a structure that contained ethanolamine, glucosamine, galactosamine, mannose, *myo*-inositol, phosphate, glycerol and stearic acid. Together with PLC studies, data obtained from these authors allowed establishing the general structure of the GPI anchor (Figure I.1).

Examples of known GPI-anchored proteins are listed in Table I.1.

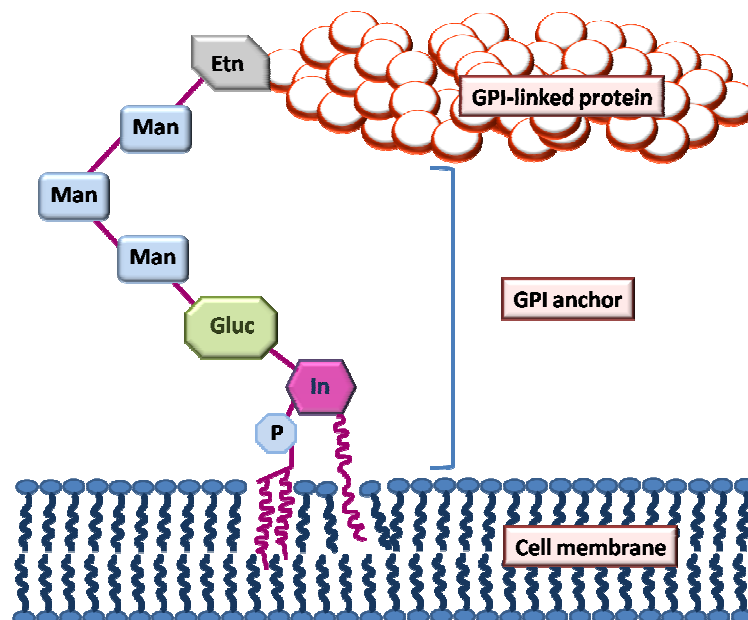


Figure I.1 – GPI-anchoring of proteins in the cell membrane. The GPI anchor consist in a phosphoethanolamine (**Etn**) that links the protein to a glycan core, composed of residues of mannose (**Man**), glucosamine (**Gluc**) and phosphoinositol (**P, In**). The latter connects to a phospholipid tail that provides a stable anchoring into the cell membrane.

Table I.1
Examples of GPI-anchored proteins in eukaryotes

Enzymes	Alkaline phosphatase, Acetylcholinesterase, 5'-Nucleotidase, Alkaline phosphodiesterase I, Trehalase, <i>Leishmania</i> surface protease PSP (gp63), Renal dipeptidase (MDP), Aminopeptidase P, NAD ⁺ glycohydrolase, Carboxipeptidase M, Carbone anhydrase IV, Silkworm aminopeptidase N, ADP-ribosyltransferase, Yeast aspartyl protease, <i>Chlorella</i> nitrate reductase
Receptors	<i>Plasmodium</i> transferrin receptor, CD14, CD16, CD48, Folate-binding protein, Urokinase receptor, CNTF receptor
Protozoal antigens	<i>Trypanosoma</i> VSG and PARP (procyclin), <i>Toxoplasma</i> surface antigens (P22, P30 and P43), <i>Giardia</i> GP49, <i>Paramecium</i> surface antigens
Mammalian antigens	Thy-1, CD55 (DAF), Ly6 family (CD59, Ly6A/E), Carcinoembryonic antigen (CEA), Qa-2, CD24
Miscellaneous	Prions (PrP ^C , PrP ^{Sc}), Squid Sgp-1 and Sgp-2, NCAM-120 (the shortest CD56), CD58 (LFA-3), <i>Dictyostelium</i> Contact site A, Mouse F3, Chick F11, Chicken axonin-1, <i>Polysphondylium</i> GP64, Grasshopper REGA-1

I.1 – Structure of the GPI anchor

The GPI anchor has a complex structure that comprises a phosphoethanolamine linker, a glycan core, and a phospholipid tail (Figure I.1).

The C-terminus of a GPI-anchored protein is linked through a phosphoethanolamine bridge to the highly conserved glycan core, comprising a mannose (α 1-2), a mannose (α 1-6), a mannose (α 1-4), a glucosamine (α 1-6), and a *myo*-inositol. A phospholipid tail attaches the GPI-anchor to the cell membrane (Figure I.2, Paulick & Bertozzi, 2008).

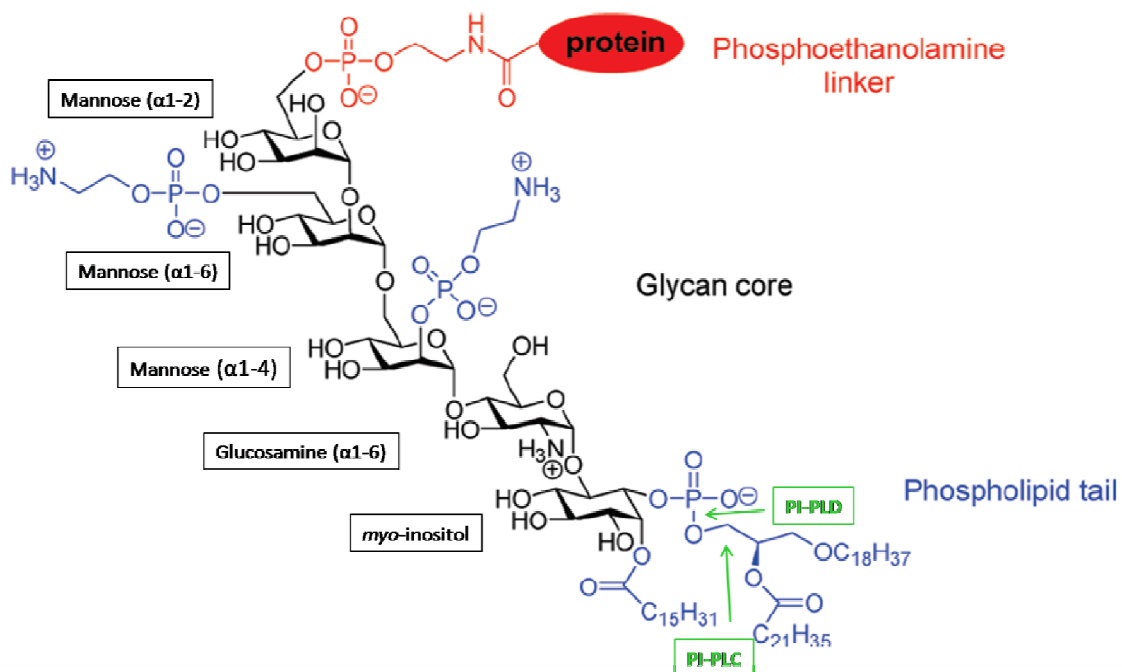


Figure I.2 – Structure of the GPI-anchor from the human erythrocyte acetylcholinesterase. Adapted from Paulick & Bertozzi, 2008. The GPI anchor is composed of three domains: the phosphoethanolamine linker (red), the glycan core (black) and the phospholipid tail (blue). Groups represented in blue are variable. Targets for phospholipase enzymes (PI-PLD and PI-PLC) cleavage are represented in green.

The phosphoinositol, glucosamine and mannose residues within the glycan core may be variously modified with phosphoethanolamine groups and other sugars (Figure I.2), rendering high diversity to these molecules.

The phosphoethanolamine side chain, attached to either the second or third mannose residue of the glycan core, is only found in higher eukaryotes, not in protozoa. GPIs from mammals, in contrast to protozoan ones, have one or two phosphoethanolamine residues, in addition to that involved in the core structure, and one phosphoethanolamine residue at the mannose site (Brewis *et al.*, 1995).

The most common side chains attached to the first mannose residue is another mannose. In both mammalian and protozoa complex side chains (like *N*-acetylgalactosamine-containing polysaccharides) are found, attached to the third mannose residue. The galactosamine residue in the core is rarely modified, except for a glycoprotein of unknown function in *Trypanosoma cruzi* (Macrae *et al.*, 2005).

The lipid anchor of the phosphoinositol ring may be a diacylglycerol, an alkylacylglycerol or a ceramide (McConville & Ferguson, 1993).

Also, many GPIs have an extra fatty acid attached to the *myo*-inositol ring that must contribute to the functions of the anchor and that renders the GPI anchor resistance to bacterial PI-PLC cleavage (Ikezawa, 2002; McConville & Ferguson, 1993; Roberts *et al.*, 1988).

The anchor of each protein is a mixture of several GPI homologues, and as such the same protein may be anchored by different GPI structures. For example, the VSG protein of *T. brucei* exhibits microheterogeneity in the number of galactose residues in the side chain (Ferguson *et al.*, 1988), and the 5'-Nucleotidase of bovine liver is anchored by a mixture of 5 homologues that differ in the substituents of the mannoside moiety (Taguchi *et al.*, 1994).

I.2 – Biosynthesis of GPI-anchored proteins

The biosynthesis of GPI precursors and the post-translational protein modifications with GPI anchor occur in the endoplasmatic reticulum (ER). During this process, the COOH terminal - (C') of the protein is split off and a new C' is combined with the ethanolamine of the GPI precursor (Ikezawa, 2002). The GPI precursor linked to the protein is transported to the Golgi complex, where further modifications in the GPI occur. After this process, GPI-anchored proteins are assumed to be transported from Golgi to the plasma membrane in the form of lipid rafts and expressed as clusters in the cell surface (Ikezawa, 2002; Kinoshita & Inoue, 2000).

GPI biosynthesis occurs in a step-wise fashion, involving several biosynthetic steps and numerous enzymes. Around 20 genes are involved in this process, and some steps are catalyzed by protein complexes consisting of 4 or more enzymes, which are listed in Table I.2 (Ferguson *et al.*, 2009).

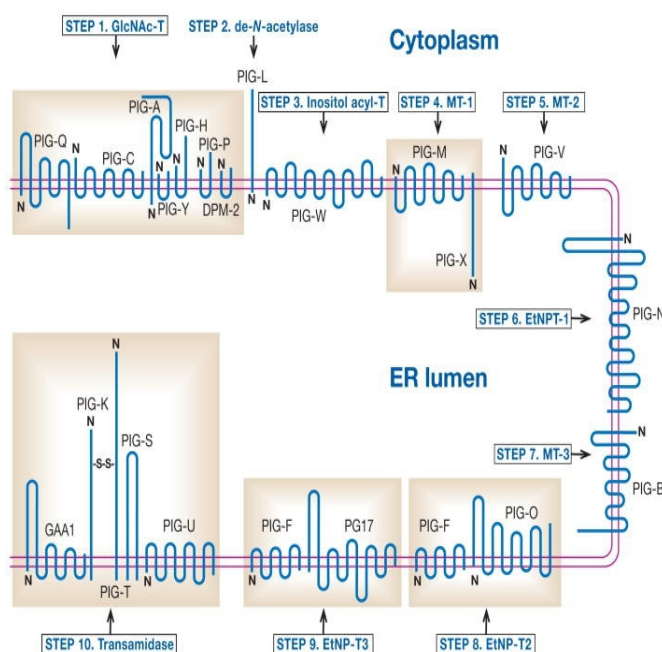
The GPI biosynthetic process starts with enzymatic attachment of an acetylated glucosamine to a phosphoinositol ring (generation of GlcNAc-PI from UDP-GlcNAc and phosphatidylinositol). Several enzymes are involved in this step, acting as a complex of at least 6 proteins (Ikezawa, 2002; Kinoshita & Inoue, 2000).

The molecule is then deacylated (generation of GlcN-PI from GlcNAc-PI), and further acylated (acylation of the inositol ring of GlcN-PI to generate GlcN-acyl-PI). Three mannose residues are then sequentially added to the GPI precursor. After the addition of the first mannose (generating Man-GlcN-acyl-PI), a modification takes place, with a phosphoethanolamine side chain (generating EtNP-Man-GlcN-acyl-PI) (Ikezawa, 2002; Kinoshita & Inoue, 2000).

Phosphoethanolamine are then added to both second and third mannose residues (generating EtNP-Man-Man-(EtNP)Man-GlcN-acyl-PI and EtNP-Man-(EtNP)Man-(EtNP)Man-GlcN-acyl-PI).

Table I.2
Enzymes involved in GPI biosynthesis

Step	Enzymes
I	PIG-A,
	PIG-H,
	UDP-GlcNAc:PI
	PIG-C,
	PIG-Q,
II	PIG-P,
	DPM-2,
	PIG-Y
	PIG-L
III	PIG-W
	PIG-M,
	PIGX
	PIG-V
	PIG-N
IV	PIG-B
	PIG-O,
	PIG-F
	GP17,
	PIG-F
V	GAA1,
	PIG-K,
	PIG-T,
	PIG-S,
	PIG-U
VI	PGAP1



(Ferguson *et al.*, 2009)

The protein is then attached to GPI, by a transamidase protein complex. Precursor peptides of GPI-linked proteins are characterized by leader sequences at both the N' and C'. The hydrophobic C' signal peptide, consisting of 20-30 aminoacid residues, is enzymatically cleaved off by the transamidase complex in order to enable covalent binding of the mature protein to the ethanolamine of the GPI anchor (Ikezawa, 2002; Kinoshita & Inoue, 2000). In most mammalian the acyl group is eliminated after protein attachment (Chen *et al.*, 1998).

The GPI biosynthetic process of both mammals and *T. brucei* is represented in Figure I.3 (Ferguson *et al.*, 2009).

The GPI biosynthesis process differs between mammals and *T. brucei* in STEP 3. In mammals, the inositol transferase acts directly on the inositol moiety of glucosamine phosphatidylinositol, then mannosylation of the glucosamine takes place. In *T. brucei* mannosylation of glucosamine phosphatidylinositol precedes to acylation of the inositol moiety. There is a unique remodeling process in which two acyl groups in the diacylglycerol moiety of phosphatidylinositol are replaced with myristol groups by sequential deacylation-reacylation (Ferguson *et al.*, 2009).

Requirements for GPI attachment are not completely identical in mammalian cells and protozoan parasites (Moran & Caras, 1994). The difference is dependent on the aminoacid triplet found at the cleavage/attachment sites. In *T. brucei*, relatively larger triplets are permitted.

Proteins to be anchored are also synthesized in the ER, and have a specific C' signal peptide for attachment to the ER membrane. Upon synthesis, the N' leader peptide is cleaved and the protein remains bound to the luminal side of the ER membrane by the C' hydrophobic peptide. The peptide is then translocated to the site of the transamidase complex. This complex cleaves the C' hydrophobic peptide, and binds the new C' to the ethanolamine head of the GPI precursor.

The C' sequence of the protein to be GPI modified comprises 4 different regions: i) an unstructured linker region of 11 residues, flexible and polar; ii) a region of small residues that includes the cleavage/attachment site; iii) a spacer sequence of moderately polar residues with intervening hydrophobic residues; iv) a hydrophobic tail (Eisenhaber *et al.*, 1998).

I.3 – Functions of GPI-anchored proteins

GPI-anchored proteins exist in every major cell type and tissue, and range in size from 12 aminoacid (as the glycopeptide Cd52) to 175kDa (protein CDw109).

GPI-anchored proteins display diverse biological functions. Many have enzymatic activities, some are involved in cell-cell contact and adhesion, others are important in the regulation of the complement cascade, and some have antigenic properties, in mammals and protozoan parasites (Paulick & Bertozzi, 2008). In *Trypanosoma brucei*, the VSG protein forms a protective coat around the parasite and acts as its major antigen (Ferguson, 1999).

Many of the GPI-anchored proteins have now been functionally characterized, but others, like the prion protein haven't been assigned a function yet.

Some GPI-anchored proteins are also believed to be involved in the embryonic development, as deficiency in the PIG-A gene, involved in the first step of GPI biosynthesis is a lethal mutation. Defects in GPI anchor biosynthesis are also lethal in yeasts (Kawagoe *et al.*, 1996; Leidich *et al.*, 1994).

I.4 – Properties of GPI-anchored proteins related to the GPI-anchor

GPIs are believed to confer the attached protein some unique properties.

The anchor may affect the overall conformation of the GPI-anchored protein, either directly, or by causing the protein to interact with the membrane. In fact, GPIs are known to alter the structure of the attached protein. The human OX7 antibody recognizes the Thy-1 glycoprotein but binding does

not occur after treatment with PI-PLC (Barboni *et al.*, 1995). Also, in *T. brucei*, an antibody that binds to the GPI-anchored protein procyclin shows remarkable reduction in affinity for the same protein lacking the lipid tail (Butikofer *et al.*, 2001).

Another interesting property of GPI-anchored proteins is the ability to transfer spontaneously to cell membranes both *in vivo* and *in vitro* – cell surface painting (Kooyman *et al.*, 1995; Medof *et al.*, 1996). The purified human decay-accelerating factor (DAF) was shown to insert onto sheep erythrocytes, and when exogenously added, it moves freely on the sheep cell surface and functions normally. This has been shown to occur with many GPI-anchored proteins, and the lipid portion of the GPI was found to be essential for cell membrane insertion (Medof *et al.*, 1996). Transgenic mice engineered to express specific GPI-anchored proteins in red blood cell alone were found to display the same proteins at the surface of vascular endothelial cells of several organs in addition to blood cells (Kooyman *et al.*, 1995). Also, human patients infected with *Trypanosoma* were found to have VSG protein from the parasite surface coat on erythrocyte membranes (Medof *et al.*, 1996). Thus, evidence indicates that GPI-anchored proteins have the ability to spontaneously transfer themselves from one cell to another.

1.4.1 – Lipid rafts – a membrane anchoring spot?

GPI-anchored proteins are believed to associate with lipid rafts, membrane microdomains enriched in glycosphingolipids, cholesterol, and certain types of lipidated proteins (Munro, 2003; Rajendran & Simons, 2005). Lipid rafts organize the plasma membrane into a series of discrete smaller domains that can serve as platforms for a variety of cellular functions, such as vesicular trafficking and signal transduction.

Lipid rafts are hypothesized to form by self-association of sphingolipids, favored by their long and mostly saturated hydrocarbons that allow them to pack tightly in a bilayer. Cholesterol molecules are believed to fill the voids between the associating sphingolipids. The presence of cholesterol may be necessary for the function and the formation of lipid rafts, as depletion of cellular cholesterol has been shown to disrupt these rafts (Ranjendran & Simons, 2005). The tight packing of sphingolipids suggests that lipid rafts are less fluid than the phospholipid bilayer. At the same time, it may allow for close packing of GPI-anchored proteins, as they show the same insolubility pattern under specific conditions as lipid rafts (Ranjendran & Simons, 2005).

The fact that signaling proteins have been associated with these lipid raft domains indicated that GPIs may be important for signal transduction (Simons & Toomre, 2000).

I.5 – GPI-anchor functions

So far, the only confirmed role of the GPI is to provide the attached protein with a stable membrane anchoring device that is resistant to most extracellular proteases and lipases (Low, 1989; Low & Saltiel, 1988). As the GPI represents an extremely complex way of protein attachment to the membrane it may serve other biological functions other than the anchoring, such as signal transduction, cell-cell communication, apical targeting, binding to bacterial toxins, prion disease pathogenesis and immune stimulation.

I.5.1 – Signal transduction

GPIs may serve as an intermediary between the exterior of a cell and internal signaling molecules and it may also allow for signal transduction by a GPI-anchored protein. The GPI does not cross the membrane but may physically interact with transmembrane proteins involved in intracellular signaling (Paulick & Bertozzi, 2008).

Antibody crosslinking between GPI-anchored proteins may affect the transduction of cellular activation or inhibition signals, resulting in Ca^{2+} fluxes, protein tyrosine phosphorylation or cytokine production. These phenomena have been associated with the anchor itself, as they are not observed when the GPI anchor is replaced by a transmembrane embedded domain (Jones & Varela-Nieto, 1998; Robinson, 1997).

I.5.2 – Cell-cell communication

Many GPI-anchored proteins are involved in signaling and cell-cell communication, such as Decay Accelerating Factor (DAF) and Thy-1. As the anchor confers them the ability to diffuse freely on the cell surface, these proteins move rapidly upon external stimuli (Low & Saltiel, 1988), and this mobility is thought to facilitate cell-cell interactions and communication.

I.5.3 – Apical targeting

Polarized cells have different membrane domains displaying different protein and lipid contents allowing for specialized functions in each domain (Schuck & Simons, 2006). In these cells, the establishment of apical and basolateral domains, separated by tight junctions, is important for asymmetric growth, directional migration and for the transport and delivery of signals and nutrients. Many of the GPI-anchored proteins are delivered to the apical domain, and the anchor has been proposed to act as an apical targeting signal. Additionally, as lipid rafts may function as platforms for the formation of apical targeting vesicles, GPIs may mediate protein-lipid raft association (Brown & Rose, 1992; Schuck & Simons, 2006).

I.5.4 – Binding to bacterial toxins

Aerolysin is a bacterial toxin secreted by *Aeromonas hydrophilia*. It is a hydrophobic protein that binds to certain sensitive cells and forms oligomers that insert into the cell membrane, forming channels and killing the cell (Diep *et al.*, 1998).

Thy-1 and contactin are receptors for aerolysin. Even though they are unrelated in function, they are both GPI-anchored, which is an important determinant of aerolysin binding (Diep *et al.*, 1998).

Some GPI-anchored proteins bind strongly to aerolysin while others do not, suggesting that the variable regions of the anchor (as sugar and phosphoethanolamine side chains) are responsible for this specificity.

The α -toxin from *Clostridium septicum* is also a pore-forming toxin that was shown to bind to GPI-anchors (Gordon *et al.*, 1999).

The fact that GPI-anchored proteins appear to concentrate in membrane microdomains such as lipid rafts may facilitate toxin concentration and oligomerization, for membrane insertion.

I.5.5 – Prion disease pathogenesis

Prion disease is characterized by the formation of insoluble protein plaques within neurons and related cells in the brain, which are associated with neurodegeneration (Taylor & Hooper, 2006). Plaque formation depends on a conformational alteration of the normal cell prion protein PrP^{C} to the pathogenic scrapie form, PrP^{SC} .

PrP^{C} is a cell-surface, GPI-linked protein expressed by a variety of cell types, but being particularly abundant in neurons (Taylor & Hooper, 2006), and thought to be involved in processes such as oxidative stress, cell signaling, copper and zinc metabolism, and in synaptic transmission.

For conversion and disease progression, the incoming PrP^{SC} has to be inserted into a contiguous membrane with PrP^{C} , in order to induce the change of PrP^{C} into PrP^{SC} , thus increasing PrP^{SC} amounts that cluster to form the plaques. Lipid rafts provide a favorable environment for conformational conversion of PrP^{C} to PrP^{SC} , by concentrating the proteins within confined regions of the membrane (Campana *et al.*, 2005; Sarnataro *et al.*, 2004). The conversion may affect signaling events involving PrP^{C} , leading to the removal of neuroprotective signals and/or the initiation of neurotoxic signals (Aguzzi, 2005).

Mice expressing an anchorless, secreted version of PrP^{C} never develop clinical prion disease, when infected with PrP^{SC} . Even though the disease was prevented, it didn't avoid PrP^{SC} plaque formation,

indicating that lack of the GPI may prevent the delivery of neurotoxic signals after PrP^{SC} plaque formation (Chesebrey *et al.*, 2005). Thus, GPI anchoring of PrP^C is essential for both the PrP^C functions and the disease propagation, as it ensures the signal transduction process.

I.5.6 – Immune stimulation

Cross-linking of GPI-anchored proteins on T lymphocytes (such as Thy-1, Ly-6 A/E, CD48 and CD59), induces T-cell mitogenesis. Ligation of GPI-anchored proteins induces an intracellular flux of Ca²⁺, an up-regulation of activation-associated cell surface proteins and the elaboration of growth promoting lymphokines (Loertscher & Lavery, 2002).

Numerous T lymphocytes cell surface membrane proteins, as Thy-1, are anchored in the membrane through a GPI (Horejsi *et al.*, 1999). Binding of the rat mAb G7 or cross linking of other Thy-1-specific mAbs induces an acute rise in Ca²⁺ concentration, IL-2 synthesis, IL-2 receptor expression and T cell proliferation (Gunter *et al.*, 1984; Kroczeck *et al.*, 1986).

PrP^C also participates in an early event of T cell activation (Cashman *et al.*, 1990). Thy-1 and PrP^C are both covalently bound to GPIs and both proteins concentrate in lipid rafts, but presenting a different distribution: Thy-1 strictly co-localizes at the center of the raft, while PrP^C is found in the semi-ordered lipid domain that separated the raft from the fluid glycerolipid domain (Madore *et al.*, 1999).

The replacement of the GPI by a transmembrane-anchoring peptide was found to abolish the activating or inhibitory potential of cell surface proteins, thus, the generation of the putative signal is related to the anchor itself (Robinson *et al.*, 1989).

I.6 – Structural significance of the GPI anchor

When expressed in cells, GPI-anchored proteins exist as heterogeneous mixtures with considerable variation in the glycan core modifications and lipid moieties (Brewis *et al.*, 1995; Ikezawa, 2002; Nosjean *et al.*, 1997; Thomas *et al.*, 1990). The same protein may be attached to anchors with different structures. These structures seem to be important for the function of the anchor however, it is not clear how this works.

The relationship between structure and function of GPIs is difficult to study due to the lack of sufficient amounts of purified anchors and anchored proteins. Well defined modifications to the GPI structure cannot be imposed using conventional biological methods, the biosynthetic enzymes are not yet fully characterized, and their disruption in cells leads to loss of the entire GPI structure (Bastisch *et al.*, 2000; Eisenhaber *et al.*, 2003; Kinoshita & Inoue, 2000; Tiede *et al.*, 1999).

The structures of GPIs of protozoan parasites such as *T. brucei*, *Leishmania* and *Plasmodium* differs from those of mammals, in respect to the decorations of the core pentasaccharide and/or lack of an associated protein, which confers parasites GPIs the properties of immunodominant epitopes (Kamena *et al.*, 2008; Schofield *et al.*, 2002).

GPI anchors exist in eukaryotic cells, from protozoa to humans. The structure of these molecules differs between groups of species and GPI's from parasites are significantly different from that of humans.

II

Apicomplexan parasites

The Apicomplexa comprise a large group of protozoans, characterized by the presence of an apical complex in at least some stages of their life cycle. These organisms are unicellular, spore-forming, unflagellated with the exception of some gametes and exclusively parasitic.

Apicomplexan parasites were first observed in 1674 by van Leeuwenhoek, who saw *Eimeria stiedai* oocysts in the gall bladder of a rabbit. By 1987, more than 4000 species, belonging to over 300 genera were included in the Apicomplexa group.

The classification of parasites into the Apicomplexa phylum has been controversial, especially because the first identification studies were based on optical microscopy.

II.1 – Biological significance of Apicomplexan parasites

Apicomplexa parasites are of high biological importance, as they are the agents of severe diseases affecting humans and animals.

Diseases include Malaria (*Plasmodium*), Toxoplasmosis (*Toxoplasma gondii*), Cryptosporidiosis (*Cryptosporidium parvum*), Cyclosporiasis (*Cyclospora cayetanesis*), Isosporiasis (*Isospora bella*) and Babesiosis (*Babesia*).

Parasites from the genus *Babesia* and *Theileria* are also responsible for cattle diseases, while *Eimeria* parasites affect mostly poultry.

II.2 – Apicomplexan parasites biology

Most members of this group have complex life-cycles that involve both asexual and sexual reproduction. In general, the invasive forms (sporozoites – Figure II.1-4) invade the host cells, where they reproduce and multiply forming merozoites. These either engage in repeated cycles of invasion and multiplication (Figure II.1-1) or differentiate into gametocytes (Figure II.1-2). These initiate the sexual reproduction, forming a zygote (cyst – Figure II.1-3). The cyst then matures and divides, forming new haploid sporozoites (Figure II.1-4).

Some variations exist in this basic life cycle according to the parasite species, including the fact that many Apicomplexa have more than one host.

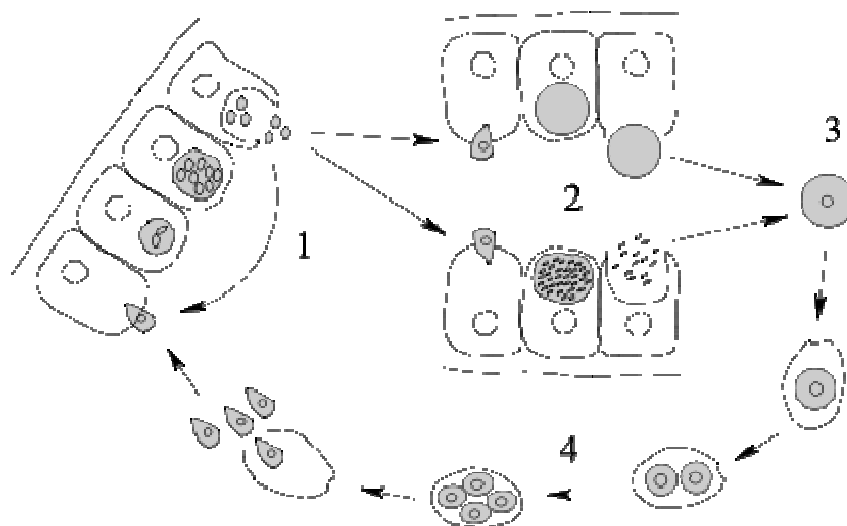


Figure II.1 – General life cycle of an Apicomplexa. From <http://commons.wikimedia.org>. 1 – merozoites; 2 – gametocytes; 3 – zygote (cyst); 4 – sporozoites.

II.2.1 – Structure of the apical complex

Apicomplexa are unicellular parasites whose cells are polarized. At the apical part of the cell there is an apical complex (Figure II.2), which is a unique structure representative of these organisms. It includes secretory organelles, such as rhoptries, micronemes and dense granules. These structures are involved in host cell invasion, as they secrete enzymes that allow the parasite to invade new cells. The tip is surrounded by a band of microtubules, called the polar ring. In some Apicomplexa there is also a funnel of rods called the conoid. This is a motile structure that contains spiral fibers that protrude during invasion. The Apicomplexa cell is surrounded by a trimembranar pellicle, underneath which lays a microtubule network, essential for parasite motility (Soldati *et al.*, 2004).

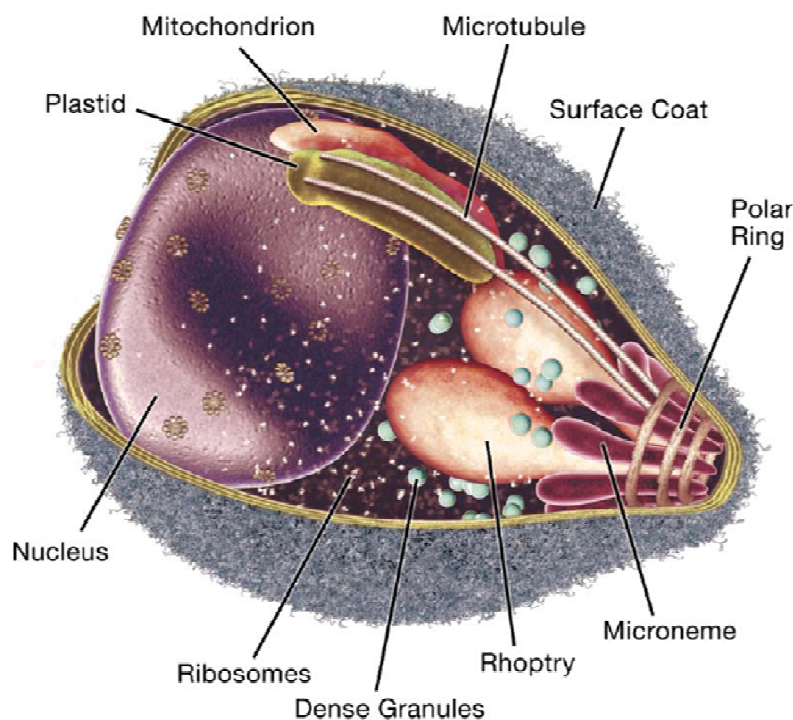


Figure II.2 – Apicomplexan apical complex. From Cowman & Crabb, 2006. The apical complex comprises the micronemes, rhoptries, dense granules and the polar ring.

Apicomplexan parasites also possess a unique structure, a single plastid, called the apicoplast, surrounded by 3-4 membranes and that functions in lipid synthesis. This is an essential organelle of secondary endosymbiotic origin that uses a bipartite leader sequences to import proteins from the nuclear genome (Waller & McFadden, 2005).

Although the basic structure of the apical complex is similar to all Apicomplexa parasites, there are species- and stage-specific variations, as not all stages of the parasite are invasive forms (zoites). For instance, the conoid is present in *Toxoplasma* and *Eimeria* parasites, and absent in *Plasmodium* and *Cryptosporidium* (Soldati *et al.*, 2004).

The apical complex is involved in crucial processes as host cell recognition and invasion.

An initial contact between the zoite (invasive form of the parasite) and the putative host cell triggers a recognition event that starts the invasion process, which occurs in a sequential fashion (Figure II.3). Briefly, contact between the apex of the parasite cell and the host cell is followed by the progressive internalization of the parasite, at the site of apical contact, which then proceeds from anterior to posterior until eventually closing the resulting vacuole around the parasite (Dubremetz *et al.*, 1998).

From the apical complex, micronemes seem to be the organelles most involved in host cell recognition and binding, secreting proteins that bind to the host cell (Dowse & Soldati, 2004; Dubremetz *et al.*, 1998). Although parasite surface proteins, like adhesins, seem to be involved in the recognition and binding to the host cell, proteins stored in the micronemes and translocated to the surface upon interaction with host cell, seem to be essential for parasite-host cell interaction. Many of these are GPI-anchored proteins. *Plasmodium falciparum* displays nine GPI-anchored

proteins at the merozoite surface, and all are potential ligands for erythrocyte binding (Cowman & Crabb, 2006).

Rhoptries have been proposed to function in the parasitophorous vacuole formation, as early in the invasion process rhoptry proteins are found at the vacuole membrane (Dubremetz *et al.*, 1998; Miller *et al.*, 1979).

Also, dense granules are exocytosed into the vacuole within minutes after it closes and their contents become associated with the vacuole contents and membrane, turning the vacuole into a metabolically active compartment (Dubremetz *et al.*, 1998).

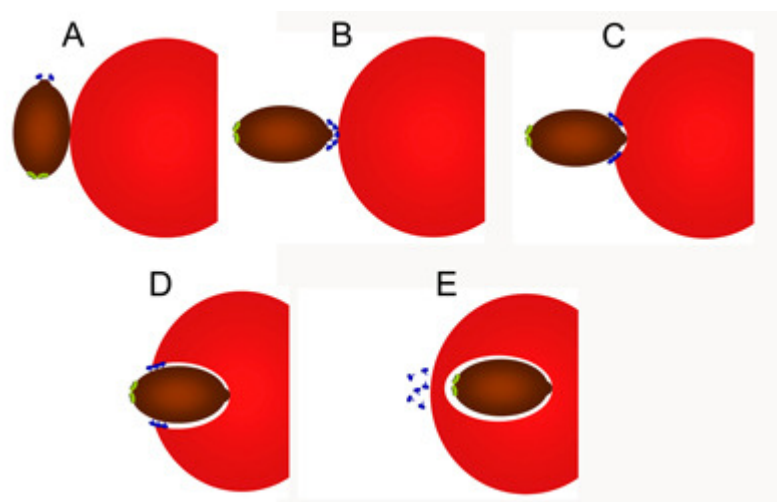


Figure II.3 – Representation of the red blood cell invasion process by *P. knowlesi* merozoites. From: <http://www.nimr.mrc.ac.uk>. **A** – Host cell recognition and initial binding. **B** – Reorientation and junction formation, through the release of high affinity adhesion protein (blue). **C, D** – Interaction of adhesins with the action-myosin motor and translocation to the posterior pole of the parasite cell. **E** – The adhesins come in contact with a rhomboid-like protease (green) on the posterior pole that releases them from the cell surface by cleavage at their transmembrane domain.

Another intriguing property of the Apicomplexa is a substrate-based gliding motility that has been found to be essential for migration through biological barriers and for host-cell invasion and egress. Some GPI-anchored adhesive proteins released, as TRAP in *Plasmodium* sporozoites and its homologue TgMIC2, in *Toxoplasma gondii*, were found to be essential for the invasion processes (Keeley & Soldati, 2004). These proteins are thought to bind to target cells, as the actin-myosin motor functions, driving the parasite forward, or into the target cell (Baum *et al.*, 2008).

III.3 – Apicomplexan parasites GPI's

Apicomplexa parasites have surface proteins anchored by GPI's, many of which are essential for parasite survival and infectivity.

GPI's from protozoans differ from those found on higher eukaryotes, in both structure and number. Protozoans seem to use these anchorage molecules much more often than higher eukaryotes. In fact most of the proteins existing at the surface of protozoans are GPI-anchored, rather than transmembrane proteins. These anchored proteins have diverse functions, like surface coating proteins, hydrolases and receptors. Additionally some are known to act in parasite protection or specific host-parasite interactions (McConville & Ferguson, 1993).

Structurally, all GPI anchors characterized contain an identical ethanolamine-phosphate-Man α 1-2Man α 1-6Man α 1-4GlcNAc1-6myo-inositol backbone. GPIs of higher eukaryotes are substituted with one or two additional ethanolamine phosphate residues, which are not observed in protozoans.

Another difference between protozoan and higher eukaryotes GPIs is that in the latter it was shown that GPI-anchored proteins accumulate in membrane microdomains as lipid rafts, while in lower

eukaryotes, there is no direct evidence that GPI-anchored proteins associate with membrane microdomains (McConville & Ferguson, 1993).

An interesting feature of some protozoal GPI anchors is that they are able to exist in a protein free state, while mammalian ones do not.

Apart from its role in protein anchoring, parasite GPIs have been found to have properties that contribute to determine the outcome of infection, in particular due to their immunogenic characteristics.

III.3.1 – Plasmodium

Plasmodium parasites cause the disease broadly known as malaria. There are around 200 species of *Plasmodium* parasites that infect animals such as birds, reptiles and mammals, most specially rodents and humans.

The parasite has a life cycle that alternates between a mosquito vector and a vertebrate host. The disease is caused by the repeated cycles of asexual reproduction in red blood cells, causing symptoms such as anemia, renal failure, fever, and so on. The deadliest form of malaria is caused by the human-malaria parasite *P. falciparum*, which induces in the parasitized red blood cells the ability to adhere to endothelial tissue, as the walls of blood capillaries. If this should happen in the brain, oxygen delivery to brain cells is hindered, and the person develops a coma that leads to death.

GPI anchors represent the major carbohydrate modification in intraerythrocytic *P. falciparum* parasites (Gowda *et al.*, 1997), and are produced in excess of what is necessary for protein attachment to the cell membrane (Gerold *et al.*, 1994).

P. falciparum synthesizes 2 forms of mature GPI anchors: a Man4-GlcN-Pi and a Man3-GlcN-Pi, in which the Pi is a 1,2-diacylglycerol and acylated inositol residues (Gerold *et al.*, 1994). Even though the intraerythrocytic stages of the parasite use both forms to anchor proteins (Schmidt *et al.*, 1998), the GPI-anchored surface proteins of *P. falciparum* MSP-1 and MSP-2 are anchored exclusively by Man4-GlcN-Pi anchors (Gerold *et al.*, 1996). Nevertheless, the parasite uses indiscriminately 5 existing different structures of the Man4-GlcN-Pi PfGPI from for protein attachment (Naik *et al.*, 2000a).

P. falciparum GPI (PfGPI) biosynthesis was shown to be essential for parasite survival, and to occur in a stage-specific manner (Naik *et al.*, 2000a). Data available on PfGPI biosynthesis revealed that in intraerythrocytic stages it occurs exclusively during maturation of early trophozoites to late trophozoites. Interference of PfGPI biosynthesis by glucosamine results in parasite growth inhibition in a dose-dependent manner, arresting trophozoite maturation (Naik *et al.*, 2003).

Besides the vital role of PfGPI as protein anchor, these molecules show an interesting feature, as they are able to immune-stimulate the vertebrate host thus contributing to the disease.

III.3.1.1 – Induction of cytokine production

PfGPIs have the ability to induce proinflammatory cytokine production by macrophages, causing symptoms that resemble malaria illness (Nebl *et al.*, 2005), and were suggested to be the dominant parasite components responsible for malaria pathogenesis. Schizont rupture leads to the release of parasite products that trigger the inflammatory cascade, resulting in acute febrile episodes (Clark *et al.*, 1981), suggesting that clinical manifestations of malaria may be mediated in part by the overproduction of proinflammatory cytokines (Miller *et al.*, 1994). Soluble parasite products were shown to activate macrophages to release TNF- α and IL-1, and PfGPI was found to be the principle mediator of this response (Schofield & Hackett, 1993).

The anchor itself stimulates the macrophages to produce TNF- α , IL-1 and IFN- γ and causes symptoms like pyrexia and hypoglycemia. The latter is related to the ability of PfGPIs to mimic the action of insulin (Elsed *et al.*, 2001).

Additionally, the endotoxin activity of major *P. falciparum* surface antigens (MSP-1 and MSP-2), released upon schizont rupture, is restricted to the GPI moiety. While protein denaturation or digestion does not affect cytokine production, treatment with the GPI cleaving Phospholipase A2 (PLA2) and others leads to a decrease of most of the proinflammatory activity (Schofield & Hackett, 1993). The bioactivity of purified GPI's is comparable to that obtained *in vivo* with live parasites (Schofield & Hackett, 1993; Schofield *et al.*, 1996).

GPIs from *P. falciparum* were found to act both against and as a substrate for cell signaling: while the glycan core activates the Protein Tyrosine Kinase (PTK) signaling, the lipid moiety acts as a second messenger for Protein Kinase C (PKC) activation (Tachado *et al.*, 1997). Although the glycan moiety can by itself activate PTK signaling, it is insufficient to induce cytokine production. The lipid moiety is only able to activate when linked to the glycan core. Thus, the simultaneous

activation of PTK and PKC signaling leads to the activation of transcription factors of the NF- κ B family, and production of proinflammatory cytokines and nitric oxide (NO).

In fact, proinflammatory cytokine induction by PfGPI was found to require the simultaneous recognition of the glycan core and the lipid moiety (Vijaykumar *et al.*, 2001).

GPIs from *P. falciparum* are recognized by Toll-Like Receptors (TLRs), namely, TLR2, and activate cytokine production via the intracellular adaptor MyD88 (Krishnegowda *et al.*, 2005), or by lectin-like receptors (Klabunde *et al.*, 2002). Recognition of PfGPI by the receptors triggers signaling pathways that lead to the induction of TNF- α , IL-6, IL-12 and NO (Zhu *et al.*, 2005). Additionally, Lu *et al.* (2006) reported phosphorylation of ERK1/2, JNK, p38, c-Jun and of the activating transcription factor-1, involved in immune signaling transduction, in PfGPI-stimulated macrophages, indicating that other pathways, such as JNK pathway, are activated in response to PfGPIs may contribute to TNF- α production in macrophages.

III.3.1.2 – Apoptosis promotion

Apoptosis also seems to play a role in malaria pathogenesis, as infection increases the number of apoptotic cells in the spleen, liver and astrocytes (Guha *et al.*, 2006; Helmby *et al.*, 2000; Potter *et al.*, 2006). Even though the numbers of apoptotic lymphocytes is similar in healthy and malaria infected patients, the number of apoptotic cells is higher in short-term cultures of lymphocytes from *P. falciparum*-infected patients (Balde *et al.*, 1995; Riccio *et al.*, 2003). Additionally, cytoadherence of infected red blood cells induces the apoptosis of endothelial cells, which may also contribute to cerebral malaria.

PfGPIs induce cardiomyocyte apoptosis *in vitro* and limited apoptosis in mouse liver and spleen tissue, when injected *in vivo*. However, it is not known if this is a direct effect of these molecules, or a result of TNF- α and NO production (Wennicke *et al.*, 2008; Wichmann *et al.*, 2007). Nonetheless,

the fact that PfGPIs up-regulate apoptotic genes and a myocardium damage marker suggests that it may have a direct effect on cardiomyocyte apoptosis, which in turn, may contribute to the lethality of malaria, as some malaria-infected patients, presenting fulminant heart failure show typical signs of cardiac myocyte apoptosis (Wennicke *et al.*, 2008).

III.3.1.3 – Induction of adhesion molecules

PfGPIs induce the expression of host adhesion molecules (ICAM-1, VCAM-1 and E-selectin) and NO production in vascular endothelial cells, in a cytokine-independent manner, not requiring TNF- α or IL-1 (Schofield *et al.*, 1996; Tachado *et al.*, 1996). NO production induced by PfGPIs involves PTK, PKC and NF-kB/c-Rel, and is blocked by anti-PfGPI antibodies (Tachado *et al.*, 1996). Adhesion molecules are of critical importance in malaria pathogenesis, as they allow for infected red blood cell sequestration in vascular endothelium, as consequently, to organ failure and cerebral malaria.

III.3.1.4 – The mosquito stage

Also in the mosquito stage, PfGPIs were showed to induce strong expression of several antimicrobial peptides and the production of NO (important for the midgut epithelium immune response to the parasite) and to dramatically reduce the number of mosquito eggs (Arrighi *et al.*, 2009; Akman-Anderson *et al.*, 2007; Lim *et al.*, 2005).

III.3.1.5 – Naturally acquired immunity to PfGPI and malaria tolerance

PfGPIs seem to act as major determinants in immune response to malaria, leading to several of the known malaria-associated clinical manifestations. In a mouse model PfGPIs were shown to have several key properties of a toxin, as they are able to induce TNF- α , IL-1 and NO production by macrophages (Schofield & Hackett, 1993; Tachado *et al.*, 1996), to up-regulate the expression of adhesion molecules in endothelial cells (Schofield *et al.*, 1996), and to induce aberrant regulation of temperature and hypoglycemia, and cause severe anemia and cachexia (Elsed *et al.*, 2001; Schofield & Hackett, 1993). All these activities were blocked with anti-PfGPI antibodies that were associated with protection from malarial acidosis, pulmonary edema, cerebral syndrome and fatality caused by infection with *P. berghei* (Schofield *et al.*, 2002).

These and other studies indicate that pro-inflammatory cytokine responses may be partially responsible for many of the clinical manifestations of severe malaria (Kwiatkowski *et al.*, 1990, 1993; Lyke *et al.*, 2004) and that immune responses against PfGPI may offer protection against symptomatic malaria in humans, known as malaria tolerance (Keenihan *et al.*, 2003).

Several field studies show that malaria infected individuals have anti-PfGPI antibodies, whose prevalence and concentration increase with age, and that this response may attenuate malaria symptoms. Highly purified PfGPIs elicited a specific IgM and IgG response and these antibodies were found in the plasma of adults and children exposed to endemic or seasonal *P. falciparum* malaria (Boutlis *et al.*, 2002; Naik *et al.*, 2000b; de Souza *et al.*, 2002).

A study that aimed at determining the anti-PfGPI antibodies load on Javanese children and adults that migrated to Papua, a malaria endemic region in Indonesia, revealed that anti-PfGPI IgGs peaked in children only after 3-4 malaria infections, while in adults peaked after the first infection. Thus, adults seem more likely to show a persistent high response. The few children that showed a strong response were less likely to experience symptoms with consequent parasitemia (Keenihan *et al.*, 2003).

Neutralization of PfGPIs by antibodies was also shown to reduce the severity of clinical attacks of malaria in the Gambia. Malaria exposed individuals had higher levels of anti-PfGPI antibodies at the end of the malaria transmission season, and were higher in children with asymptomatic infections. Although antibodies seemed to be rapidly boosted by clinical malaria infection, children under 2 years old were seronegative for anti-PfGPI antibodies, even during an acute infection. Nonetheless, this population showed relatively low levels of anti-PfGPI antibodies, that are rather short-lived, indicating that the provided immunity to clinical symptoms may be only transient (de Souza *et al.*, 2002).

In a Kenyan population, malaria-immune adults maintained high levels of anti-PfGPI antibodies, while children at risk of developing clinical malaria and anemia had lower antibody levels (Naik *et al.*, 2000b). This study established an association between anti-PfGPI levels and protection from severe malaria anemia and fever in children.

A study in Senegal associated the anti-PfGPI response to protection against cerebral malaria, as patients with confirmed cerebral malaria had significantly lower levels of anti-PfGPI IgGs than those with mild malaria (Perraut *et al.*, 2005).

In Mandang, Papua New Guinea, a region where malaria transmission is intense, the prevalence and concentration of anti-PfGPI antibodies also increased with age in asymptomatic children and adults (Boutlis *et al.*, 2002). The antimalarial treatment reduced the anti-PfGPI antibody response in children and adolescents within 6 weeks. PfGPI elicited both IgG and IgM responses. While the IgG response is more persistent in adults and of the IgG3 subclass (Boutlis *et al.*, 2003), IgM response is generally of shorter duration and coincides with infection.

All these studies indicate that natural immunity and tolerance to malaria in endemic or seasonal malaria regions includes the development of anti-PfGPI antibodies, which levels increase with age of the host, and that are in fact associated with the attenuation of the disease.

III.3.2 – Toxoplasma

The medical significant species of this genus is *Toxoplasma gondii* that causes a disease called toxoplasmosis. This parasite has the cat as its definite host, but it can be carried by nearly all warm-blooded animals (Montoya & Liesenfeld, 2004).

Acute infections can be asymptomatic, but often give flu-like symptoms in the early-acute stages, that fade in a few days/months, when the parasite enters in a latent stage. Although this stage is mainly asymptomatic, it can develop in immunocompromised patients, as HIV infected people, causing severe encephalitis that leads to death (Luft & Remington, 1992). In pregnant women *T. gondii* infection can cause congenital infectious diseases (Desmonts *et al.*, 1981).

In the developmental stage in which virulent tachyzoites differentiate into bradyzoites, where disease propagation is observed, there is an overexpression of free GPIs from *T. gondii* (TgGPIs) and tachyzoites-specific GPI-anchored proteins (Azzouz *et al.*, 2006).

TgGPIs are essential for parasite viability (Wichroski & Ward, 2003), and exist in two precursor forms, both of which are able to bound to proteins in animal cells (Zinecker *et al.*, 2001), while in human cells, one of them remains in the free, unbound form. This precursor has an additional glucose residue linked to the N-acetylglucosamine side branch and has immunogenic properties, as the ability to induce high levels of TNF- α production (Azzouz *et al.*, 2006; Striepen *et al.*, 1997).

III.3.2.1 – GPI-anchored *T. gondii* surface antigens

In the host, *T. gondii* parasites interact with the immune system, and the initial interaction is achieved by the parasite's surface antigens. *T. gondii* has a superfamily of GPI-anchored antigens that includes the surface antigen (SAG) family and the SAG-unrelated antigen (SUSA) family (Boothroyd *et al.*, 1997; Jung *et al.* 2004; Lekutis *et al.*, 2001). It has been proposed that SAG proteins could regulate virulence by eliciting the host immune response, be involved in immune evasion, and participate in invasion of a variety of host cells. SUSA proteins (SUSA1 and SUSA2) were found to be highly expressed in the chronic form of the parasite, as well as to interact with the host immune system (Pollard *et al.*, 2008).

III.3.2.2 – TgGPI induction of cytokine production

Apart from surface antigens, the GPI anchor itself seems to elicit immune responses from the host. TgGPIs dominate the surface of *T. gondii* tachyzoites and were shown to be involved in the pathogenicity of the parasite infection. TgGPIs are able to activate tyrosine kinase in macrophages (Debierre-Grockiego *et al.*, 2007a), and to induce TNF- α production from macrophages, through the activation of the transcription factor NF- κ B (Debierre-Grockiego *et al.*, 2003). The glycan cores and lipid moieties from the anchor were sufficient to stimulate macrophages.

MyD88, an adaptor molecule in the TLR signaling for NF- κ B activation was proved to be essential for the response to *T. gondii* (Scanga *et al.*, 2002). Additionally, in hamsters, *T. gondii* isolated GPI anchors were found activate the immune receptor TLR4, while the glycan core and the lipid moieties activate both TLR2 and TLR4 *in vivo* (Debierre-Grockiego *et al.*, 2007a). Deficiency on MyD88 impairs TNF- α production by macrophages upon stimulation with *T. gondii* GPI anchors, confirming that this immunogenicity depends on TLR recognition of the TgGPI, MyD88 signaling and NF- κ B-dependent transcription of TNF- α .

Debierre-Grockiego and co-workers (2009) also found that the GPI anchor from *T. gondii* up-regulates MHC class I and class II molecules on the surface of both unstimulated and IFN-gamma-stimulated primary murine macrophages, thus increasing antigen presentation to CD8(+) lymphocytes.

III.3.2.3 – Apoptosis promotion

T. gondii is able to promote and inhibit apoptosis in the infected cells. It can render the infected cell resistant to programmed cell death induced by several apoptotic signals (Carmen *et al.*, 2006; Goebel *et al.*, 1999, 2001; Luder & Gross, 2005; Nash *et al.*, 1998; Orlofsky *et al.*, 1999, 2002; Payne *et al.*, 2003). The increased apoptosis of immune cells after *T. gondii* infection may suppress the response to the parasite (Khan *et al.*, 1996; Mordue *et al.*, 2001; Wei *et al.*, 2002).

As high levels of apoptosis are accompanied by hyperinduction of proinflammatory cytokines, TgGPIs were proposed to contribute to the development of apoptosis. TgGPI failed to block apoptosis induction in human-derived cells. Interestingly it also did not promote apoptosis, since markers of apoptosis, as caspase 3/7 activation, phosphatidylserine expression at the cell surface or DNA strand breaks were not observed in the presence of TgGPIs, suggesting that these are not involved in survival or apoptosis of host cells (Debierre-Grockiego *et al.*, 2007b).

III.3.3 – Other Apicomplexan parasites

Not much data is yet available regarding GPIs of other Apicomplexan parasites. Some studies performed so far focus on GPI-anchored proteins identified, and only a few on the properties of the anchor itself.

III.3.3.1 – *Eimeria*

This is the genera of the Apicomplexa that comprises more species. These parasites infect the gastrointestinal tract of a broad range of vertebrate animals, including birds such as chickens and mammals like goats and rodents.

As in *T. gondii*, the surface of *Eimeria* parasites is coated with a family of developmentally expressed GPI-anchored surface antigen (SAG) proteins that are involved in processes such as invasion and are most likely recognized by the host immune system. Surface GPI-anchored proteins are differentially expressed in the different invasive stages (oocyst, sporozoite, merozoite) in a way that is not related to antigen-switching but rather to a co-expression of the TgGPI-anchored proteins in each parasite (Tabarés *et al.*, 2004). Apart from this, not much is known about the GPI-anchors and GPI-anchored proteins of *Eimeria* parasites, and the role they play in infection.

III.3.3.2 – *Babesia*

Babesia parasites infect blood cells and are characterized by their round, rod or abstract shape and lack of any mobility structures, as cilia or flagella. This group includes several species, many of which can infect humans, causing Babesiosis, although this disease occurs mainly in cattle. These parasites are transmitted by ticks.

In cattle, *Babesia bovis* parasites stimulate the inducible nitric oxide synthase (iNOS) transcription and NO production by peripheral blood macrophages (Stich *et al.*, 1998), as well as the production of inflammatory and regulatory cytokines, such as IL-1 β , IL-12 and TNF- α (Shoda *et al.*, 2000).

A lipid fraction of *B. bovis* infected erythrocytes also induces NO production by bovine macrophages, indicating that GPI molecules from this parasite may signal for immune responses in the host (Shoda *et al.*, 2000).

Data obtained so far in Apicomplexa parasites GPIs have focused mainly in *Plasmodium* and *Toxoplasma*, due to their medical significance. Nonetheless, GPIs seem to be important in general for Apicomplexa parasite survival, and GPI-anchored proteins have been identified in a few more parasites. Further studies should provide more information about the role of these molecules in the infection by these parasites.

IV

GPI anchors as therapeutic targets

The Apicomplexa parasite to which most attention has been paid in terms of therapeutics and vaccine development has been the malaria causing *Plasmodium*. However, antigenic-based vaccination for parasite clearance has proved to be difficult and in most cases, ineffective, which may increase the selective pressure upon the parasite that is able to mount escape mechanisms, such as antigenic variation, immune evasion and others. Thus, inducing sterilizing immunity does not seem to be a feasible objective for anti-malaria vaccination that should rather aim to prevent or reduce morbidity and mortality (Schofield, 2007).

An alternative strategy is based on anti-toxic vaccination, as the development of anti-toxic vaccines should confer immunization against parasite products that cause host pathology, and avoiding parasite killing, which might not be a necessary objective. Also, malaria morbidity and fatalities were shown to have a toxic basis, which argues in favor of the development of anti-toxic vaccines (Schofield, 2007).

Malaria toxin-induced cytokines (TNF- α , IL-1 and IL-6) may contribute to a systemic anti-inflammatory cascade which results in malaria-associated symptoms, as renal failure and pulmonary edema, shock, etc. Additionally, organ-specific disease may result from local release of toxin by sequestered parasites (Schofield, 2007). Thus, preventing toxin-induced symptoms should help alleviate the burden of the disease.

PfGPIs are parasite toxins that were shown to be the major TNF- α and IL-1 inducer, sufficient to kill mice when inoculated (Schofield & Hackett, 1993). Also, mice immunized against Man4-PfGPIs were protected from severe malarial pathology, including cerebral syndrome, acidosis, pulmonary edema and fatality (Schofield *et al.*, 2002), thus revealing in an animal model the efficacy of a prototype anti-toxic vaccine.

In human populations from malaria endemic and seasonal areas, malaria exposed individuals were shown to carry anti-PfGPI antibodies in the serum, indicating that naturally acquired immunity to malaria involves the recognition of parasite GPIs and the subsequent establishment of anti-PfGPI response. Importantly, these antibodies were frequently associated with disease protection, and, up

to date, most data associate anti-PfGPI antibodies to clinical protection against malarial anemia (Keenihan *et al.*, 2003; Naik *et al.*, 2000b; Perraut *et al.*, 2005; de Souza *et al.*, 2002).

It is important that vaccination strategies do not impair the development of naturally acquired immunity.

Naturally acquired immunity to malaria ameliorates the clinical impact of infection and maintains the host/pathogen balance, although it does not confer resistance to infection (Schofield, 2007). Accordingly, mechanisms of acquired immunity to the disease are set both earlier and more easily than anti-parasite immunity that is difficult to achieve and partially effective, even though ongoing exposure to the parasite is necessary to maintain immunity (Schofield & Mueller, 2006).

Blocking PfGPI toxicity was shown to protect the host from the disease without killing the parasite, which also contributes to reducing the selective pressure upon the parasite (Schofield, 2007).

These parasite toxins are responsible for proinflammatory cytokine induction and cytoadherence promotion, thus contributing to several malaria-associated symptoms. The fact that individuals tolerant to malaria disease were shown to have an anti-GPI based naturally acquired immune response to malaria and that blocking of GPI toxicity reduces the severity of malaria-associated symptoms suggest that parasite GPIs are good candidates for therapeutics and vaccine development. Also as anti-GPI responses were shown not to interfere with parasitemia indicates that an anti-GPI-based vaccination strategy would not impose an extended selective pressure upon the parasite, which is important to avoid vaccination failure.

Based on these data, Schofield (2007) proposed a strategy based on conjugating chemically synthetic PfGPI glycan to an immunogenic carrier protein of parasite or bacterial origin.

GPIs exist in the surface of eukaryotic cells, being widely distributed among the Eukarya Kingdom. These molecules anchor proteins with diverse functions, including enzymes, receptors, surface antigens and others, thus being essential for numerous processes vital for survival. Even though its basic structure is similar in all molecules characterized so far, several differences, especially regarding the modifications in the side chains of the glycan core and the lipid moiety were found in GPI molecules. Different structures are present in the same organism, but critical differences are found between organisms, especially between higher eukaryotes and protozoans. These differences extend also to the biosynthetic pathway, indicating that these molecules and their biosynthesis may provide suitable therapeutic targets for controlling protozoan parasites infections.

Particularly in malaria disease, naturally acquired immunity and tolerance to the disease is, at least in part, associated with anti-PfGPIs antibodies. These attenuate the clinical manifestations of the disease without affecting the parasite development, thus reducing the selective pressure upon the parasite and consequently decreasing selection of mutant parasites. Anti-GPI vaccination strategies thus promise a more reliable and effective protection than antigen-based vaccines.

V

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